This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS

- TEXT CUT OFF AT TOP, BOTTOM OR SIDES

- FADED TEXT

- ILLEGIBLE TEXT

- SKEWED/SLANTED IMAGES

- COLORED PHOTOS

- BLACK OR VERY BLACK AND WHITE DARK PHOTOS

- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.

(51) International Patent Classification⁷: C12N 15/31, 15/09, 1/21, A61K 39/108 // (C12N 1/21, C12R 1:19)

(21) International Application Number: PCT/KR99/00555

(22) International Filing Date:
15 September 1999 (15.09.1999)

(25) Filing Language: English

(26) Publication Language: English

(71) Applicant (for all designated States except US): MOGAM BIOTECHNOLOGY RESEARCH INSTITUTE [KR/KR]; 341, Pojung-ri, Koosung-myon, Yongin-si, Kyonggi-do 449-910 (KR).

(72) Inventors; and
(75) Inventors/Applicants (for US only): PARK, Eun, Jeong [KR/KR]; 322-1007, Woosung Apt., Sangrok-maeul, Jungja-dong, Pundang-ku, Sungnam-si, Kyonggi-do 464-010 (KR). KIM, Jang, Seong [KR/KR]; 902 Ikjoo Apt., Yeonmoo-dong, Jangan-ku, Suwon, Kyonggi-do 440-240 (KR). CHANG, Jihoon [KR/KR]; 553-517, Jayang 3-dong, Kwangjin-ku, Seoul 143-193 (KR). YUM, Jungsun [KR/KR]; 115-502, Chunggu Apt., Hansol-maeul, Jungja-dong, Pundang-ku, Sungnam-si,

Kyonggi-di 463-010 (KR). CHUNG, Soo-il [US/KR]; 112-902, Hyundae Apt., Hyoja-chon, Seohyun-dong, Pundang-ku, Sungnam-si, Kyonggi-do 463-480 (KR).

(74) Agent: LEE, Han-Young; Seowon Building, 8th floor, 1675-1, Seocho-dong, Seocho-gu, Seoul 137-070 (KR).

(81) Designated States (national): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— With international search report.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: NOVEL DETOXIFIED MUTANTS OF ESCHERICHIA COLI HEAT-LABILE ENTEROTOXIN

(57) Abstract: The present invention relates to detoxified and immunologically active proteins ("mutant LTs") having mutated amino acid sequences of heat-labile enterotoxin of E. coli, DNA sequences encoding the mutant LTs, recombinant expression vectors comprising the DNAs, recombinant microorganisms transformed with the recombinant expression vectors, process for preparing the mutant LTs and pharmaceutical application of the said protein as immunogenic antigens for vaccination and as adjuvants for antibody production. In contrast to wild-type LT, the mutant LTs did not induce any toxic activities. The mutant LTs elicited high and comparable levels of anti-LT antibodies when delivered either intragastrically or intranasally, inducing systemic and local responses in serum and fecal extracts. Thus, they might be useful for the development of a novel diarrheal vaccine in humans and animals. In addition, the antibody production ability using mutant LTs as an adjuvant may be effective for prevention and treatment of various diseases.

NOVEL DETOXIFITED MUTANTS OF
Escherichia coli HEAT-LABILE ENTEROTOXIN

5 BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to detoxified and
10 immunologically active proteins("mutant LTs"), more
specifically, to mutant LTs having mutated amino acid
sequences of heat-labile enterotoxin of *E. coli,* DNA
sequences encoding the mutant LTs, recombinant expression
vectors comprising the DNAs, recombinant microorganisms
15 transformed with the recombinant expression vectors,
process for preparing the mutant LTs and pharmaceutical
application of the said proteins as immunogenic antigens
for vaccination and as adjuvants for antibody production.

20 Description of the Prior Art

Enterotoxigenic *Escherichia coli*("ETEC") strain
causes diarrheal disease in humans and animals due to
production   of   toxin   such   as   heat-labile
25 enterotoxin("LT")(see: Spangler, B.D., Microbiol. Rev.,
56:622-647(1992)). LT is a multimeric protein composed of
two functionally distinct domains: an enzymatically active
A subunit("LTA") of ~30,000daltons with ADP-ribosylating
activity,   and   a   pentameric   B   subunit("LTB")   of
30 ~11,600daltons   that   contains   GM1(momosialoganglioside)
receptor-binding site(see: Bäckström, M. et al., Mol.
Microbiol., 24:489-497(1997)). Upon thiol reduction, the A
subunit dissociates into two polypeptide chains, i.e.,
A1(Mr, 23,000 daltons) and A2(Mr, 6,000daltons)(see: Tsuji,
35 T. et al., J. Biol. Chem., 260:8552-8558(1985); Grant C.C.R.
et al., Infect. Immun., 62:4270-4278(1994)). The A1
subunit, in particular, intoxicates eucaryotic cells by

catalyzing ADP-ribosylation of Gs, a GTP-binding protein that regulates the levels of the second messenger cAMP(see: Guerrant, R.L. et al., Infect. Immun., 10:320-327(1974); Field, M. et al., N. Engl. J. Med., 321:800-806(1989)).

5   The resulting increase in cAMP level causes secretion of water and electrolytes into the small intestine through interaction with two cAMP-sensitive ion transport mechanisms including (i) NaCl co-transport across the brush border of villous epithelial cells and (ii) electrogenic

10 Na^+-dependent Cl^- secretion by crypt cells(see: Guidry, J.J. et al., Infect. Immun., 65:4943-4950(1997)).

Both the cholera toxin("CT") from *Vibrio cholerae* and heat-labile enterotoxin from ETEC belong to the most potent mucosal adjuvants and immunogens known to date by oral and

15 other mucosal routes, via which most of antigens are unable to induce immune responses(see: Jackson, R.J. et al., Infect. Immun., 61:4272-4279(1993); Takahashi, I. et al., J. Infect. Dis., 173:627-635(1996)). However, their toxicities have precluded their clinical use in humans(see: Douce, G.

20 et al., Proc. Natl. Acad. Sci., USA, 92:1644-1648(1995)). One approach to overcome the problem of toxicity is the generation of genetically detoxified derivatives of LT(see: Lobet, Y. et al., Infect. Immun., 59:2870-2879(1991); Dickson, B.L. and Clements, J.D., Infect. Immun., 63:1617-

25 1623(1995)) and CT(see: Fontana, M.R. et al., Infect. Immun., 63:2356-2360(1995); Yamamoto, S. et al., Proc. Natl. Acad. Sci., USA, 94:5267-5272(1997b)) by site-directed mutagenesis of amino acids which are located on the ß-strand that constitutes the 'floor' of NAD-binding cavity.

30 The most important factor for immunogenicity is shown to be the ability to bind to the receptor on eucaryotic cell(see: Nashar, T.O. et al., Proc. Natl. Acad. Sci., USA, 93:226-230(1996)). In fact, a non-binding mutant of the B subunit of LT was found to be non-immunogenic(see: Guidry,

35 J.J. et al., Infect. Immun., 65:4943-4950(1997)). In addition, another group found that the ADP-ribosylating activity is unnecessary for immunogenicity because nontoxic

derivatives of LT obtained by site-directed mutagenesis of the A subunit retained the immunological properties of the wild-type LT(see: Pizza, M. et al., J. Exp. Med., 180:2147-2153(1994)).

5   The attempt to define the role of ADP-ribosylating activity in adjuvanticity of LT has generated conflicting results.   For example, it was reported that a nontoxic derivative of LT(LTE112K) when co-administered with keyhole limpet hemocyanin(KLH) by an oral route in mice, lacked the

10   adjuvant properties, thus suggesting that the adjuvanticity of LT is linked to its ADP-ribosylating activity(see: Lycke, N. et al., Eur. J. Immunol., 22:2277-2281(1992)). However, more recently, the adjuvant activity of the LTE112K was found to be identical to that of the LT holotoxin when

15   delivered with influenza virus surface antigen by an intranasal route(see: Verweij, W.R et al., Vaccine, 16:2069-2076(1998)). On the other hand, other investigators showed that another LT derivatives, LTK63, lacking enzymatic activity and toxicity was still able to elicit

20   antibody responses against the co-administered antigen in mice immunized orally, intranasally, or intravaginally(see: Di Tommaso, A. et al., Infect. Immun., 64:974-979(1996); Giuliani, M.M. et al., J. Exp. Med., 187:1123-1132(1998); Marchetti, M. et al., Vaccine, 16:33-37(1998)).

25   Under the circumstances, the present inventors, based on the findings that detoxified LT derivatives may induce antibody responses, tried to explore an efficient immunogenic antigen and mucosal adjuvant for vaccination, which can be applied for the development of a mucosal

30   vaccine as well as a novel diarrheal vaccine for humans and animals.

SUMMARY OF THE INVENTION

35   In accordance with the present invention, the inventors have made an effort to develop detoxified and immunologically active proteins("mutant LTs") by the site-

directed mutagenesis of heat-labile enterotoxin(LT) of *E. coli*. The inventors cloned full length DNA coding for the LT, mutated the A subunit by site-directed mutagenesis and constructed expression vectors comprising the DNAs of
5 mutated LT and recombinant microorganisms transformed with the recombinant expression vectors. Further, the inventors found that the recombinant mutant LTs can be applied as an active ingredient for diarrheal vaccine and adjuvant for mucosal vaccine.
10

The first object of the invention is, therefore, to provide detoxified and immunologically active proteins which have mutated amino acid sequences of heat-labile enterotoxin of *E. coli*.
15 The second object of the invention is to provide DNA sequences encoding the said mutant LTs.

The third object of the invention is to provide recombinant expression vectors comprising the said DNAs.

The fourth object of the invention is to provide
20 recombinant microorganisms transformed with the said recombinant expression vectors.

The fifth object of the invention is to provide a process for preparing recombinant mutant LTs from the said microorganisms.
25 The sixth object of the invention is to provide a diarrheal vaccine comprising an active ingredient of mutant LT.

The seventh object of the invention is to provide a novel use of the mutant LT as an adjuvant for mucosal
30 vaccine.

BRIEF DESCRIPTION OF THE INVENTION

The above and the other objects and features of the
35 present invention will become apparent from the following description given in conjunction with the accompanying drawings, in which:

Figure 1 is a photograph showing SDS-PAGE of a wild-
type and mutant LTs produced and
isolated from recombinant *E. coli*.

Figure 2 is photographs showing the changes in CHO-K1
cell morphology after treatment with
wild-type or mutant LTs.

Figure 3 is a photograph showing ADP-
ribosyltransferase activity of
wild-type LT, LTS63Y and LTΔ
110/112 analyzed by SDS-PAGE
followed by autoradiography.

Figure 4 is a graph showing the amount of
intracellular cAMP of CHO cells
measured by an enzyme immunoassay
system after treatment with wild-
type or mutant LTs.

Figure 5 is graphs showing anti-LT secretary IgA,
serum IgG and IgA antibody responses on
intragastric or intranasal immunization.

Figure 6 is graphs showing anti-urease secretary IgA,
serum IgG and IgA antibody responses on
intragastric immunization.

Figure 7 is graphs showing anti-urease secretary IgA,
serum IgG and IgA antibody responses on
intranasal immunization.

Figure 8A is a graph showing anti-urease serum IgG
subclass antibody responses on
intragastric immunization.

6

Figure 8B is a graph showing anti-urease serum IgG
subclass antibody responses on
intranasal immunization.

5 DETAILED DESCRIPTION OF THE INVENTION

The present inventors first isolated a DNA fragment
including heat-labile enterotoxin(LT) gene from
enterotoxigenic *E. coli* K88ac strain of porcine origin and
10 thus cloned into pBluescript KS(-) vector to generate
pBlueKS-/rLT for site-directed mutagenesis. Site-directed
mutagenesis was performed on single-stranded DNA of
pBlueKS-/rLT vector. As a result, two vectors that have
mutated DNA sequences encoding amino acids of proposed ADP-
15 ribosyltransferase active center of LT were generated: the
first one is 'pBlueKS-/LTS63Y' wherein serine residue at
position 63 of LT is substituted with tyrosine; and, the
other one is 'pBlueKS-/LT⊿110/112' wherein glutamic acid
residues at positions 110 and 112 are deleted.

20 Amino acids at positions 58 to 72 are shown to be
folded in a β-strand followed by α-helix, which form the
NAD-binding site and amino acids Arg^7, His^{44}, Ser^{61} Glu^{110} and
Glu^{112} have been shown to be important for enzymatic
activity. Up to date, the modified residues of the LTA
25 subunit are Arg^7, Ser^{63}, Glu^{110}, Glu^{112} or Ala^{72}.

The inventors have substituted Ser^{63}, the essential
amino acid for NAD-binding and catalytic activity of LT,
with Tyr residue having a bulky side chain of phenolic ring.
Thus, it was expected to efficiently block NAD-binding in
30 slightly modified LT structure. The inventors have also
deleted Glu^{110} and Glu^{112} in LTA subunit which are important
for enzymatic activity, and have studied for immunogenicity
to LT itself and immune-eliciting property against
coadministered antigens. The double deletion mutant of the
35 invention was expected to completely eliminate enzymatic
activity of LT.

In describing the mutant LTs of the present invention, LTS63Y is employed to mean mutated LT in which serine residue at position 63 is substituted with tyrosine; and, "LT⊿110/112" is employed to mean mutated LT in which glutamic acid residues at positions 110 and 112 of LT are deleted, respectively.

pBlueKS-/LTS63Y and pBlueKS-/LT⊿110/112, were transformed into E. coli Top 10F'. Each of the transformants thus prepared was designated as 'Escherichia coli Top 10F'-pBlueKS-/LTS63Y' and 'Escherichia coli Top 10F'-pBlueKS-/LT⊿110/112' and deposited with the Korean Collection for Type Cultures(KCTC) located at KRIBB #52, Oun-dong, Yusong-gu, Taejon 305-333, Republic of Korea, an international depository authority as accession Nos. KCTC 0648BP and KCTC 0649BP, respectively.

E. coli Top 10F' transformed with either pBlueKS-/LTS63Y or pBlueKS-/LT⊿110/112 was grown in LB broth containing 100 μg/ml of ampicillin and the mutant LTs were purified from the cultures using immobilized D-galactose column and FPLC Superdex 200 column.

Since the mobilities of mutant LTs were identical to those of the wild-type LT on SDS-PAGE analysis, the molecular weight of the mutant LT subunits were presumed to be identical to those of wild-type LT. These results suggest that mutant LTs consisting of A and B subunits retain the AB_5 conformation similar to wild-type LT.

The ability of mutant LTs to induce morphological changes in cultured Chinese hamster ovary-K1(CHO-K1) cells(ATCC, USA) was tested. The morphological changes in the CHO-K1 cells were used to detect the toxic activity of mutant LTs(see: Grant, C.C.R. et al., Infect. Immun., 62:4270-4278(1994)). As little as 100ng/ml of LT induced longitudinal growth of approximately 90% of the CHO-K1 cells, a response previously shown to be dependent upon adenylate cyclase-induced increases in cAMP(see: Guerrant, R.L. et al., Infect. Immun., 10:320-327(1974)). However, the cells treated with each of the mutant LT at the level

of 10μg/ml showed no morphological changes of the CHO-K1 cells.

ADP-ribosyltransferase activity was determined as the ability to catalyze the transfer of labeled ADP-ribose from [adenylate-^{32}P]NAD to the 41kDa G protein in CHO-K1 membrane(see: Locht, C. et al., Infect. Immun., 55:2546-2553(1987)). In general, A1 subunit of LT is known to catalyze ADP-ribosylation of the membrane-bound substrate G proteins. When membrane proteins from CHO-K1 cells were incubated with wild-type LT in the presence of [adenylate-^{32}P]NAD, it specifically ADP-ribosylated the Mr 41,000 proteins, which correspond to the α subunits of the GTP binding Gs protein. In contrast, no ADP-ribosylation of this protein was detected in reaction mixtures incubated with the same amounts of LTS63Y or LT⊿110/112. This result was identical to that of the negative control treated without toxins. Therefore, the substitution of Tyr^{63} for Ser^{63} or deletion of Glu^{110} and Glu^{112} in A1 subunit appeared to cause changes in structural integrity of NAD binding crevice that may be important for enzymatic activity of LT.

In addition, the levels of cAMP were determined in CHO cells treated with CT, LT, or mutant LTs. The addition of CT or LT caused about 10-fold higher levels of cAMP production than that of untreated cultures. On the other hand, changes in cAMP levels in LTS63Y- or LTΔ110/112-treated cells were barely detectable. These data showed that the presence of A subunit of wild-type LT(accurately LTA1 subunit) is necessary for an increase in the intracellular cAMP concentration and the mutant derivatives, LTS63Y and LTΔ110/112, devoid of enzymatic activity, are unable to form cAMP.

The enterotoxicity experiments of mutant LTs examined using a mouse ileal loop test strongly indicate that the mutant LTs possess negligible enterotoxicity in vivo.

In the immunogenic ability of mutant LTs, the mice immunized with LTS63Y or LT⊿110/112 contained high and

comparable levels of anti-LT antibodies in sera and fecal extracts compared with those immunized with wild-type LT. Moreover, both of the intragastric and intranasal immunizations using mutant LTs could be an effective method

5 for inducing antibody responses of vaccination.

The ability of mutant LTs to act as a mucosal adjuvant was assessed by intragastric immunization in mice. LT⊿110/112 was effective as a mucosal adjuvant on intragastric immunization by inducing high levels of

10 mucosal and systemic antibody responses to the coadministered antigens such as *H. pylori* whole cell lysate or urease. The ability of mutant LTs to function as a mucosal adjuvant was also assessed by intranasal immunization in mice. Intranasal administration of LTS63Y

15 demonstrated the sensitiveness in inducing mucosal immunigenecity and adjuvanticity. Mice immunized intranasally by coadministration of urease antigen and LTS63Y showed strong mucosal and systemic anti-urease responses including urease-specific secretary IgA, serum

20 IgG and IgA antibodies.

The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

25

Example 1: Expression and Purification of mutant LTs

Example 1-1: Plasmid construction and mutagenesis

30 A 1.5Kb *BamHI* DNA fragment including LT gene from enterotoxigenic *E. coli* K88ac strain of porcine origin was cloned into pBluescript KS(-) vector(Stratagene, USA). The resulting vector, designated as pBlueKS-/rLT, was used for site-directed mutagenesis.

35 Site-directed mutagenesis was performed on single-stranded DNA prepared from *E. coli* CJ236 transformed with pBlueKS⁻/rLT according to the method of MutanK kit(Takara

Biomedicals, Japan). The sequence of oligonucleotides used for the substitution and deletion of amino acids were 5'-ATATGATGACGGATATGTTTCCACTTACCTTAGTTTGAGAAGTGCTCACTTG-3'(SEQ ID NO:1) and 5'-AGGCGTATACAGCCCTCACCCATATCAGGTTTCTGCGTTAGG

5   TGGAATACCAT-3'(SEQ ID NO:2), respectively. As a result, serine residue at position 63 was substituted with tyrosine and glutamic acid residues at positions 110 and 112 were deleted, respectively.

These residues are in proposed ADP-ribosyltransferase

10 active center of LT and their substitutions or deletions have been shown to inactivate ADP-ribosyltransferase activity and enterotoxicity(see: Domenighini, M. et al., Mol. Microbiol., 14:41-50(1994)).

Amino acids at positions 58 to 72 are shown to be

15 folded in a β-strand followed by α-helix, which form the NAD-binding site and amino acids Arg^7, His^{44}, Ser^{61} Glu^{110} and Glu^{112} have been shown to be important for enzymatic activity. Up to date, the modified residues of the LTA subunit are Arg^7(to Lys), Ser^{63}(to Lys), Glu^{110}(to Asp),

20 Glu^{112}(to Asp or Lys) or Ala^{72}(to Arg)(see: Lobet, Y. et al., Infect. Immun., 59:2870-2879(1991); Fontana, M.R. et al., Infect. Immun., 63:2356-2360(1995); Di Tommaso, A. et al., infect. Immun., 64:974-979(1996); Douce, G. et al., Infect. Immun., 63:2821-2828(1997); Marchetti, M.M. et al., Vaccine,

25 16:33-37(1998); Tsuji, T. et al., FEBS Letters, 292:319-321(1991); Lycke, N. et al., Eur. J. Immunol., 22:2277-2281(1992); Verweij, W.R. et al., Vaccine, 16:2069-2076(1998); Giuliani, M.M. et al., J. Exp. Med., 187:1123-1132(1998)).

30 The substitution of Ser^{63}, the essential amino acid for NAD-binding and catalytic activity of LT, with Tyr residue having a bulky side chain of phenolic ring was expected to efficiently block NAD-binding in slightly modified LT structure. Deletion of Glu110 and Glu112,

35 which are located at the putative ADP-ribosyltransferase active center was also expected to completely eliminate enzymatic activity of LT.

The changes of DNA sequences were confirmed using Sequenase Version 2.0 sequencing kit(Amersham Life Science, USA) and each of mutants thus prepared was designated as 'pBlueKS-/LTS63Y' and 'pBlueKS-/LT∆110/112', respectively.

Example 1-2: Expression and purification of the recombinant mutant LTs

pBluescript KS(-) vectors containing the mutant LT gene which comprises the 160bp 5'-noncoding region, 1.2kb coding region and 197bp 3'-noncoding region, pBlueKS-/LTS63Y and pBlueKS-/LT∆110/112, were transformed into *E. coli* Top 10F'(Invitrogen, USA). Each of the transformants thus prepared was designated as '*Escherichia coli* Top 10F'-pBlueKS-/LTS63Y' and '*Escherichia coli* Top 10F'-pBlueKS-/LT∆110/112' and deposited with the Korean Collection for Type Cultures(KCTC) located at KRIBB #52, Oun-dong, Yusong-gu, Taejon 305-333, Republic of Korea, an international depository authority as accession Nos. KCTC 0648BP and KCTC 0649BP, respectively.

E. coli Top 10F' transformed with either LTS63Y or LT∆110/112 was grown in LB broth containing 100 µg/ml of ampicillin and the mutant LTs were purified from the cultures. The cells were harvested by centrifugation, resuspended in TEAN buffer(50mM Tris-HCl, pH 7.5, 0.2M NaCl, 1mM EDTA and 3mM NaN$_3$), and lysed with a microfluidizer(Microfluidics Corporation, USA). The lysates were clarified by centrifugation and then filtered using 0.45µm membrane(Micro Filtration Systems, Japan) prior to chromatography on an immobilized D-galactose column (Pierce, USA) (see: Uesaka, Y. et al., Microb. Pathog. 16:71-76(1974)). Bound proteins were eluted with 0.3M galactose in TEAN buffer. Holotoxin(AB$_5$) was separated from the free B-subunit pentamer by size exclusion chromatography using FPLC Superdex 200 column(Pharmacia, Sweden).

The homogeneity of LTS63Y and LT∆110/112 was confirmed by SDS-polyacrylamide gel electrophoresis(see:

Figure 1). In Figure 1, lanes 1 and 2 represent wild-type LTs; lanes 3 and 4, LTS63Ys; and, lanes 5 and 6, LTΔ 110/112s, respectively, and the arrow 1 is a position of LT holotoxins and LTB subunit pentamers; arrow 2, LTA subunit;

5 and, arrow 3, LTB subunit monomer. Each lane received 10μg of the proteins and samples in lanes 2, 4 and 6 were heated to 95℃ for 5min in the presence of β-mercaptoethanol, while samples in lanes 1, 3 and 5 were loaded without denaturation by heating and adding β-mercaptoethanol. When

10 the purified mutant LTs were analyzed without denaturation, two protein bands appeared: one band with the size of 70-100kDa corresponding to the holotoxin and LTB pentamers; and, the other band with the size of about 30kDa corresponding to the LTA subunit. When the purified mutant

15 LTs were boiled for 5min with β-mercaptoethanol, the holotoxins were dissociated into two bands of about 30 and 11kDa, corresponding to the A and B subunits of LT, respectively. Since the mobilities of mutant LTs were identical to those of the wild-type LT, the molecular

20 weight of the mutant LT subunits were presumed to be identical to those of wild-type LT.

These results suggest that the innate structure of the A subunit associated with pentameric B subunits of LT is not affected by substitution of tyrosine for Ser[63] or

25 deletion of Glu[110] and Glu[112] residues on NAD-binding pocket. Moreover, it was demonstrated that the binding ability of the B subunit of mutant LTs to G_{M1} ganglinoside was similar to that of the normal B subunit using a G_{M1}-ELISA, and mutant LTs were reacted with anti-LT antibody in Western

30 blot analysis. These results imply that mutant LTs retain the AB_5 conformation similar to wild-type LT.

<u>Example 2</u>: Enzymatic and Biologic Characterization of mutant LTs

35

<u>Example 2-1</u>: Cell elongation assay

The ability of mutant LTs to induce morphological changes in cultured Chinese hamster ovary-K1(CHO-K1) cells(ATCC, USA) was tested as described previously(see: Guerrant, R.L. et al., Infect. Immun., 10:320-327(1974);

5   Grant, C.C.R. et al., Infect. Immun., 62:4270-4278(1994)). CHO-K1 cells were incubated for 24hr as monolayer cultures in minimal essential medium alpha(MEM-α)(Gibco-BRL, USA) supplemented with 10% fetal bovine serum(FBS) in humidified and 5% CO_2 atmosphere at 37℃. The cells were washed once

10   with Hanks's balanced salt solution(HBSS) (Sigma, USA) and then incubated with 0.1% trypsin for 5min. After centrifugation, they were washed once, and then resuspended in the growth medium. To each well of a 48-well tissue culture plate, the same numbers of CHO-K1 cells(10^4 in

15   200μl volume per well) were allowed to adhere for 4hr prior to the addition of the toxin dilutions and then incubated in humidified and 5% CO_2 atmosphere at 37℃ for 24hr. The cells were then washed with phosphate-buffered saline(PBS), fixed with methanol, and stained with 0.04% Trypan Blue

20   Solution(Gibco-BRL, USA). After staining, the cells were washed, air dried, and analyzed for morphological changes by light microscopy.

The morphological changes in the CHO-K1 cells were used to detect the toxic activity of mutant LTs(see: Grant,

25   C.C.R. et al., Infect. Immun., 62:4270-4278(1994)). As little as 100ng/ml of wild-type LT induced longitudinal growth of approximately 90% of the CHO-K1 cells, a response previously shown to be dependent upon adenylate cyclase-induced increases in cAMP(see: Guerrant, R.L. et al.,

30   Infect. Immun., 10:320-327(1974)). However, the cells treated with mutant LTs at the level of 10μg/ml showed no morphological changes of the CHO-K1 cells(see: Figure 2 and Table 1). In Figure 2, A shows toxin-untreated CHO-K1 cells; B, CHO-K1 cells treated with 100ng/ml of wild-type

35   LT; C, CHO-K1 cells treated with 10μg/ml of LTS63Y; and, D, CHO-K1 cells treated with 10μg/ml of LT$\varDelta$110/112, respectively.

Table 1. Comparison of biologic and enzymatic activities of
wild-type LT, LTS63Y and LT⊿110/112

Toxin assessed	Cell elongation[a]	Ileal loop test[b]
PBS	10%<	negative
Wild-type LT	90%> at 100ng	positive at 100ng
LTS63Y	10%< at 10μg	negative at 100μg
LT⊿110/112	10%< at 10μg	negative at 100μg

a: 10[4] of CHO-K1 cells were cultured with 100ng of wild-type LT or
   10μg of each mutant LT for 24hr and a positive toxin effect in the
5 CHO-K1 cells was defined as elongation of >20% of the cells
   according to published criteria(see: Guerrant, R.L. et al., Infect.
   Immun. 10:320-327 (1974))

b: The enterotoxicity of mutant LTs was examined using on ileal loop
10 test, where mice were anesthetized, and 100μl of PBS containing
   100ng of wild-type LT or 100μg of each mutant LT was injected into
   an ileal loop. Loops were examined 18hr later and the ratio of
   fluid to length was defined as positive when the ratio was
   >40μl/cm(see: Fujita, K. et al., J. Infect. Dis. 125: 647-655
15 (1972))

Example 2-2: ADP-ribosyltransferase activity test

20   For the preparation of crude membranes, CHO-K1 cells
   were maintained in monolayer culture by serial passage in
   MEM-α medium supplement with 10% FBS(see: Locht C. et al.,
   Infect. Immun., 55:2546-2553(1987)). The cells were
   detached from the flask, washed once in PBS(pH 7.2) and
25 then lysed in a homogenizer (Weaton, USA). The supernatant
   from these lysates was collected following centrifugation
   at 1,000xg for 20 min and further centrifuged at 18,000xg
   for 7 min to obtain microsomal or membrane pellets. The
   washed membrane pellet was resuspended in 50mM Tris-
30 HCl(pH8.0) at a concentration of 1mg of protein per ml and
   stored at -70°C until use.
        ADP-ribosyltransferase activity was determined as the
   ability to catalyze the transfer of labeled ADP-ribose from
   [adenylate-^{32}P]NAD to the 41kDa G protein in CHO-K1
35 membrane(see: Locht, C. et al., Infect. Immun., 55:2546-
   2553(1987)). Reaction mixtures (100μl) containing 32μM
   [adenylate-^{32}P]NAD(2μCi)(NEN, USA), 50 mM Tris-HCl(pH 8.0)

containing 10mM thymidine, 100mM ATP, 20mM DTT, 100μM GTP, 50μg of CHO-K1 membrane proteins, and 10μg of wild-type or mutant LTs were incubated at 37℃ for 30min. Reactions were terminated by the addition of 1ml of ice-cold 50mM
5   Tris-HCl(pH 8.0) and the membranes were sedimented by centrifugation(15,000xg, 7min) at 4℃. The membrane pellet was resuspended in ice-cold Tris-HCl(pH 8.0) and sedimented once more by centrifugation before being solubilized in 50μl of electrophoresis sample buffer(67mM Tris-HCl, pH 6.8,
10 2% SDS, 0.03% bromophenol blue, and 10% glycerol) containing 5% β-mercaptoethanol. The samples were heated to 95℃ for 5min and then analyzed by SDS-PAGE and autoradiography.

In general, the A1 subunit of LT is capable of
15 binding NAD and catalyzing the ADP-ribosylation of membrane-bound Gs, a GTP-binding regulatory protein associated with adenylate cyclase(see: Spangler, B.D., Microbiol. Rev., 56:622-647(1992)). The consequence is a sharp increase in cAMP production resulting in excessive
20 accumulation of salts and water in the intestinal lumen(see: Field, M. et al., N. Engl. J. Med., 322:800-806(1989)). The A subunit of LT is known to catalyze ADP-ribosylation of the membrane-bound substrate G proteins.

Figure 3 shows ADP-ribosyltransferase activity of LT,
25 LTS63Y and LT𝛥110/112 analyzed by SDS-PAGE in a 12.5% gel followed by autoradiography. In Figure 3, lane 1 represents a reaction mixture incubated without toxins; lane 2, with 10μg of wild-type LT; lane 3, with 10μg of LTS63Y; and, lane 4, with 10μg of LT𝛥110/112, respectively,
30 and the arrow denotes the position of the Mr-41,000 band corresponding to the Gs protein.

As shown in Figure 3, when 50μg of membrane proteins from CHO-K1 cells were incubated with wild-type LT in the presence of [adenylate-^{32}P]NAD, it specifically ADP-
35 ribosylated the Mr-41,000 proteins, which correspond to the α subunits of the GTP binding Gs proteins. In contrast, no ADP-ribosylation of this protein was detected in reaction

mixtures incubated with the same amounts of LTS63Y or LTΔ 110/112. This result was identical to that of the negative control treated without toxins in lane 1. Therefore, the substitution of Tyr[63] for Ser[63] or deletion of Glu[110] and Glu[112]

5 in A subunit did cause changes in structural integrity of NAD binding crevice that may be important for enzymatic activity of LT.

Example 2-3: Measurement of intracellular cAMP accumulation
10
    CHO cells(ATCC, USA) were maintained in MEM-α medium supplemented with 10% FBS in a 24-well plate at a concentration of 5x10^4 cells per well, grown to near confluency, and incubated in MEM-α containing FBS and 1mM

15 3-isobutyl-1-methylxanthine (IBMX) for 30min prior to addition of toxins(see: Grant, C.C.R. et al., Infect. Immun., 62:4270-4278(1994)). Either cholera toxin(CT), cholera toxin B subunit(CTB), trypsin-activated wild-type LT, LTS63Y, or LTΔ110/112 was added to each well and the

20 plates were incubated for 18h. The cells were washed three times with PBS and intracellular cAMP was extracted by adding 200μl of 50mM HCl to each well and placing the plates in -70℃ deep freezer for 20min. cAMP was measured with a Biotrak cAMP enzyme immuno-assay(EIA)

25 system(Amersham Life Science, USA) according to the manufacturer's instructions.
    As a result, the levels of cAMP were determined in CHO cells treated with CT, CTB, LT, LTS63Y, or LTΔ 110/112(see: Figure 4). As shown in Figure 4, the addition

30 of CT or LT to reach a concentration of 50ng/ml caused about 10-fold higher levels of cAMP production than those of untreated cultures. On the other hand, cAMP formation in cultures treated with CTB, LTS63Y or LTΔ110/112 was undetectable even at a concentration as high as 5μg/ml. In

35 Figure 4, 'S' and 'D' denote LTS63Y and LTΔ110/112, respectively, and the gray bars represent treatments of 500ng of CT or LT and 5μg of CTB, S or D; and, black bars,

17

50ng of CT or LT and 500ng of CTB, S or D. Results are shown as mean titers and error bars indicate standard deviations from the mean.

These data showed that the presence of wild-type LTA subunit(accurately LTA1 subunit) is necessary for an increase in the intracellular cAMP concentration and the mutant derivatives, LTS63Y and LTΔ110/112, devoid of enzymatic activity, are unable to form cAMP.

Example 2-4: Assessment of toxicity using mouse ileal loops

The enterotoxicity of mutant LTs was examined using a mouse ileal loops test(see: Yamamoto, S. et al., J. Exp. Med., 185:1203-1210(1997)). Groups of mice were anesthetized, and different doses of each toxin were injected into ileal loops(LT, 100ng or 1μg per mouse; and, mLT, 10μg or 100μg per mouse) of individual mice. The mice were sacrificed 18hr after the injection, and the fluid content of the ileal loops was determined; values of more than 40μl/cm were considered positive (indicative of toxicity).

One hundred nanogram of wild-type LT induced significant fluid accumulation in small intestine, while no fluid accumulation was observed in the loop treated with thousand-fold higher levels(100μg) of mutant LTs(see: Table 1). These data strongly indicate that the mutant LTs possess negligible enterotoxicity in vivo.

Example 3: Immunological Characterization

Example 3-1: Mucosal immunogenicities of mLTs

Six-week-old female Balb/c mice were purchased from Charles River(Japan). The mucosal immunogenicities of LTS63Y and LTΔ110/112 were tested via two immunization routes. Groups of mice were immunized intragastrically with 25μg of LTS63Y or LTΔ110/112 four times on days 0, 7,

14 and 21 or intranasally with 2μg of LTS63Y or LT∆110/112
on days 0, 7 and 14(see: Takahashi, I. et al., J Infect.
Dis., 173:627-635(1996)). The control groups received PBS
alone. The serum and fecal antibody titers to LT were
determined using samples prepared on day 7 following the
last immunization(see: Figure 5). Figure 5 shows anti-LT
secretary IgA, serum IgG and IgA antibody responses on
intragastric(white   bar)   or   intranasal(black   bar)
immunization, where 1 represents PBS treatment; 2, wild-
type LT treatment; 3, LTS63Y treatment; 4, LT∆110/112
treatment.

        As shown in Figure 5, the mice immunized with LTS63Y
or LT∆110/112 contained high and comparable levels of
anti-LT antibodies in sera and fecal extracts compared with
those immunized with wild-type LT. The LTS63Y was slightly
more immunogenic than LT∆110/112 on both intragastric and
intranasal administration. On the other hand, titers of
anti-LT in the serum or fecal extracts of mice intranasally
immunized with wild-type or mutant LTs were slightly higher
than those observed in mice intragastrically administered.
Intranasal immunization offers several advantages compared
with other immunization route: lower doses of proteins are
required to induce antibody responses, which means lower
cost for vaccine production(see: Yamamoto, S. et al., Proc.
Natl.   Acad.   Sci.,   USA,   94:5267-5272(1997)).   When
administered intranasally, only 6% of the quantity of
mutant LT used in intragastric immunization was required to
elicit slightly higher levels of secretary IgA responses
and this dose also effectively induced systemic IgG and IgA
antibody responses. Thus, intranasal immunization using
mutant LT could be an effective method for vaccination in
humans and animals.

Example 3-2: Mucosal adjuvanticity of mutant LTs

        To test a mucosal adjuvanticity, mice were immunized
either intragastrically by 125μg of H. pylori urease

together with 25μg of LTS63Y or LTΔ110/112 on days 0, 7,
14 and 21 or intranasally by 20μg of the same antigen
together with 2μg of LTS63Y or LTΔ110/112 as an adjuvant
on days 0, 7 and 14. Fecal extracts and serum samples were
5   collected on day 7 following the last immunization and the
appearance of either mucosal or systemic antibody responses
was monitored using ELISA.

LT- and urease-specific antibodies were measured with
a G_{M1} capture enzyme-linked immunosorbent assay(G_{M1}-ELISA)
10 and direct ELISA, respectively, as described
previously(see: Spiegel, S. J. Cell. Biochem., 42:143-
152(1990); Douce, G. et al., Infect. Immun., 65:2821-
2828(1997)). Plates were coated with 150ng of G_{M1}(Sigma,
USA) per well of a 96-well EIA/RIA plate(Costar, USA) for a
15 G_{M1}-capture ELISA, and then incubated at 37℃ for 1hr.
Plates were washed three times with PBS containing 0.05%
Tween 20("PBST") and blocked with 2.5% skim milk(Difco,
USA) in PBST at 37℃ for 1hr. After washing with PBST
three times, 100ng of wild-type LT was added into wells and
20 plates were incubated for 1hr at 37℃ and washed three
times with PBST. In case of a direct ELISA, plates were
coated with 1μg of urease per well incubated for 1hr at
37℃ with horseradish peroxidase (HRP)-conjugated anti-
mouse antibodies specific for mouse immunoglobulinG (IgG)
25 (1:5000), IgA (1:2000) (KPL, USA) or IgG1, IgG2a or IgG2b
(1:2000) (Biosource, USA). After washing six times with
PBST, bound antibody was visualized by addition of
3,3'5'5'-tetramethylbenzidine (TMB). The absorbance at 450
nm was determined and ELISA titers were recorded as the
30 highest dilution of serum which gave an absorbance value
above the level measeured in preimmune samples.

Mucosal adjuvanticity of LTS63Y and LTΔ110/112 by
intragastric immunization

35

The ability of mutant LTs to act as a mucosal
adjuvant was assessed by intragastric immunization in

mice(see: Figure 6: 1, PBS treatment; 2, 125μg urease
treatment; 3, 125μg urease and 25μg LT treatment; 4, 125μg
urease and 25μg LTS63Y treatment; 5, 125μg urease and 25μg
LT△110/112 treatment).

5   As shown in Figure 6, mice immunized intragastrically
with *H. pyroli* urease alone showed no significant levels of
antibodies   to   the   antigen.   Mice· immunized   by
coadministration of urease and LT△110/112 produced high
levels of urease antibody responses comparable to those
10   immunized with wild-type LT, while mice immunized by
coadministration of the antigen and LTS63Y showed no
significant sero-conversion.   Altering the dose of LT△
110/112 to 10 or 125μg did not cause any change in the
immune response to urease.   LTS63Y did not show any
15   significant adjuvanticity to the urease, even when 125μg
was coadministered with the urease.

        To test the adjuvanticity of LT△110/112 with other
antigens, whole cell lysate of *H. pylori* was used as an
antigen and it was observed that LT△110/112 also induced
20   antibody responses to whole cell lysate.   These data
suggest that LT△110/112 effectively functions as a mucosal
adjuvant on intragastric immunization by inducing   strong
mucosal and systemic antibody responses to coadministered
antigens such as *H. pylori* whole cell lysate or urease.

25   To further assess the immunologic response induced by
LT△110/112   following   intragastric   immunization,   IgG
subclass responses were determined by ELISA(see: Figure 8A:
1, PBS treatment; 2, 125μg urease treatment; 3, 125μg
urease and 25μg LT treatment; 4, 125μg urease and 25μg
30   LTS63Y treatment; 5, 125μg urease and 25μg LT△110/112
treatment).

        As shown in Figure 8A, urease antibody responses
enhanced by LT△110/112   were largely restricted to
IgG1(gray bars), rather than   IgG2a(white bars) or
35   IgG2b(black bars) subclass antibody in sera, and similar
antibody patterns were observed using wild-type LT as an
adjuvant.

Generally, CT elicits adjuvant responses by inducing antigen-specific CD4+ T cells secreting interleukin 4(IL-4), IL-5, IL-6 and IL-10 that correlated directly with serum IgG1 and IgG2b subclass responses in mice orally immunized

5   with protein Ag and CT as adjuvant(see: Marinaro. M. et al., J. Immunol., 155:4621-4629(1995). It was reported that mutant CT(S61F), despite lack of ADP-ribosyltransferase activity with resultant cAMP induction, elicits serum IgG1 and IgG2b subclass Ab responses when administrated

10 intranasally(see: Yamamoto, S. et al., Proc. Natl. Acad. Sci., USA, 94:5267-5272(1997) or subcutaneously(see: Yamamoto, S. et al., J. Exp. Med., 185:1203-1210(1997). On the other hand, oral immunization with LT promotes IgG1, IgG2a and IgG2b, which are supported by a mixed CD4+ Th1-

15 and Th2-type responses associated with IFN-γ, IL-4, IL-5, IL-6 and IL-10 production(see: Takahashi, I. et al., J. Infect. Dis., 173:627-635(1996). In the results of present invention, intragastric administration of LT∆110/112 or wild-type LT as an adjuvant induced predominant IgG1 Ab

20 responses as shown in Figure 8A. This result is not consistent with the typical IgG subclass responses induced by wild-type LT and rather resembles the responses induced by CT activating CD4+ Th2-type cells(see: Marinaro, M. et al., J. Immunol., 155:4621-4629(1995). In contrast, LTS63Y

25 induced IgG1, IgG2a and IgG2b antibody responses to *H. pylori* urease on intranasal immunization. The similar result is also observed by wild-type LT eliciting IgG1, IgG2a and IgG2b subclass responses, which are supported by a mixed CD4+ Th1- and Th2-type response(see: Takahashi, I.

30 et al., J. Infect. Dis., 173:627-635(1996)). Therefore, the mutant LTs of this invention, LTS63Y and LT∆110/112, induced distinct IgG subclass responses, depending on immunization routes.

35 <u>Mucosal adjuvanticity of LTS63Y and LT∆110/112 by intranasal immunization</u>

The ability of mutant LTs to function as a mucosal adjuvant was also assessed by intranasal immunization in mice. Intranasal administration of wild-type or mutant LTs demonstrated the sensitiveness in inducing mucosal

5   immunigenecity and adjuvanticity(see: Figure 7: 1, PBS treatment; 2, 20μg urease treatment; 3, 20μg urease and 2μg wild-type LT treatment; 4, 20μg urease and 2μg LTS63Y treatment; 5, 20μg urease and 2μg LT△110/112 treatment).

Mice immunized intranasally by coadministration of

10 urease antigen and LTS63Y showed high levels of mucosal and systemic anti-urease responses including urease-specific secretary IgA, serum IgG and IgA antibodies, which were equivalent to the responses observed when wild-type LT was used as an adjuvant. However, mice immunized by

15 coadministration of urease and LT△110/112 showed lower level of antibodies to urease, similar to those levels induced by urease alone. When 0.2μg of LTS63Y was used, antibody responses to urease in both fecal extracts and sera were lower by a factor of approximately 10, but when

20 6μg of LTS63Y was used, no increase in antibody responses to urease was induced.

In addition, as shown in Figure 8B, LTS63Y induced IgG1(gray bars), IgG2a(black bars) and IgG2b(white bars) subclass antibody responses to *H. pylori* urease, and these

25 results were quite different from those IgG subclass responses(predominant IgG1) resulted from the intragastric immunization with the mutant LT△110/112(see: Figure 8B: 1, PBS treatment; 2, 20μg urease treatment; 3, 20μg urease and 2μg LT treatment; 4, 20μg urease and 2μg LTS63Y treatment;

30 5, 20μg urease and 2μg LT△110/112 treatment). Therefore, the mutant LTs of this invention, LTS63Y and LT△110/112 induced district IgG subclass responses, depending on immunization routes.

As described above, the mutant LTs of this invention

35 have shown different abilities to act as mucosal adjuvant according to the route of administration. This suggests that different mutant forms of LT may require different

immunization routes for adequate adjuvanticity, and that specific immunization route may elicit specific mechanisms of up-regulation of immune responses, independent of cAMP levels. Thus, selection of the route of immunization may be

5   critical for determining the mucosal adjuvant activity of mutant LTs. Mucosal vaccines delivered into the nasal tract provide several advantages. For example, lower doses of antigen and adjuvant are required to induce effective antibody responses when compared to intragastric

10   immunization, which can decrease the cost of vaccination.

As clearly illustrated and demonstrated as above, the present invention provides detoxified and immunologically active proteins(mutant LTs). The mutant LTs was expressed

15   from recombinant expression vectors, pBlueKS-/LTS63Y and pBlueKS-/LTΔ110/112 that contain mutated DNA sequences encoding amino acids in the ADP-ribosyltransferase active center. In contrast to wild-type LT, both of the LTS63Y and LTΔ110/112 did not induce any toxic activities. Both

20   of the mutants elicited high and comparable levels of anti-LT antibodies when delivered either intragastrically or intranasally, inducing systemic and local responses in serum and fecal extracts. Thus, they might be useful for the development of a novel diarrheal vaccine in human and

25   animals. In addition, the antibody production ability using LTS63Y and LTΔ110/112 as adjuvants against *H. pylori* urease may be effective for prevention and treatment of various diseases. The adjuvant activity of these mutants might be very useful to develop an effective mucosal

30   vaccine component.

24

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO: Mogam Biotechnology Research Institute
#341, Pojung-ri, Kusung myun, Yongin si, Kyonggi-do 449-910,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: *Escherichia coli* Top10F'-pBlueKS'/LTS63Y	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0648BP

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **Jul 27 1999.**

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on   and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

V. INTERNATIONAL DEPOSITARY AUTHORITY

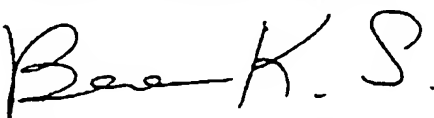| Name: Korean Collection for Type Cultures	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):
Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	BAE, Kyung Sook. Director Date: **Aug 02 1999**

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO: Mogam Biotechnology Research Institute
#341, Pojung-ri, Koosung-myun, Yongin-si, Kyonggi-do 449-910,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli Top10F'—pBlueKS'/LT Δ 110/112 .	KCTC 0649BP

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **Jul 27 1999.**

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on   and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Korean Collection for Type Cultures	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):
Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	BAE, Kyung Sook, Director Date: **Aug 02 1999**

<u>WHAT IS CLAIMED IS</u>:

1. A detoxified and immunologically active protein having an amino acid sequence of heat-labile enterotoxin of *E. coli*, in which position 63 or positions 110 and 112 is mutated in a site-directed manner.

2. A detoxified and immunologically active protein having an amino acid sequence of heat-labile enterotoxin of *E. coli*(SEQ ID NO:3), in which serine residue at position 63 is substituted with tyrosine.

3. A DNA sequence(SEQ ID NO:4) encoding the detoxified and immunologically active protein of claim 2.

4. A recombinant expression vector pBlueKS-/LTS63Y which comprises the DNA of claim 3.

5. *Escherichia coli* Top10F'-pBlueKS-/LTS63Y(KCTC 0648BP) transformed with the recombinant expression vector pBlueKS-/LTS63Y of claim 4.

6. A detoxified and immunologically active protein having an amino acid sequence of heat-labile enterotoxin of *E. coli*(SEQ ID NO:5), in which glutamic acid residues at positions 110 and 112 are deleted.

7. A DNA sequence(SEQ ID NO:6) encoding the detoxified and immunologically active protein of claim 6.

8. A recombinant expression vector pBlueKS-/LT⊿ 110/112 which comprises the DNA of claim 7.

9. *Escherichia coli* Top10F'-pBlueKS-/LT⊿110/112(KCTC 0649BP) transformed with the recombinant expression vector pBlueKS-/LT⊿110/112 of claim 8.

10. A process for preparing the detoxified and immunologically active protein(mutant LT) of claim 1 which comprises the steps of culturing a recombinant microorganism transformed with an expression vector comprising a DNA encoding the mutant LT and isolating the recombinant mutant LT from the culture.

11. The process for preparing the detoxified and immunologically active protein of claim 10, wherein the recombinant microorganism is *Escherichia coli* Top10F'-pBlueKS-/LTS63Y(KCTC 0648BP) or *Escherichia coli* Top10F'-pBlueKS-/LT⊿110/112(KCTC 0649BP).

12. A diarrheal vaccine comprising an active ingredient of the detoxified and immunologically active protein of claim 1 and pharmaceutically acceptable carrier.

13. A mucosal adjuvant comprising an active ingredient of the detoxified and immunologically active protein of claim 1.
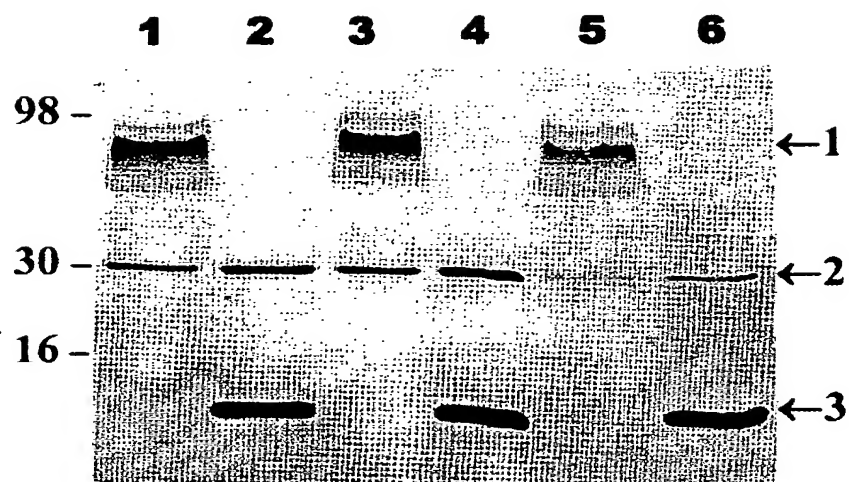
1/9

Fig. 1
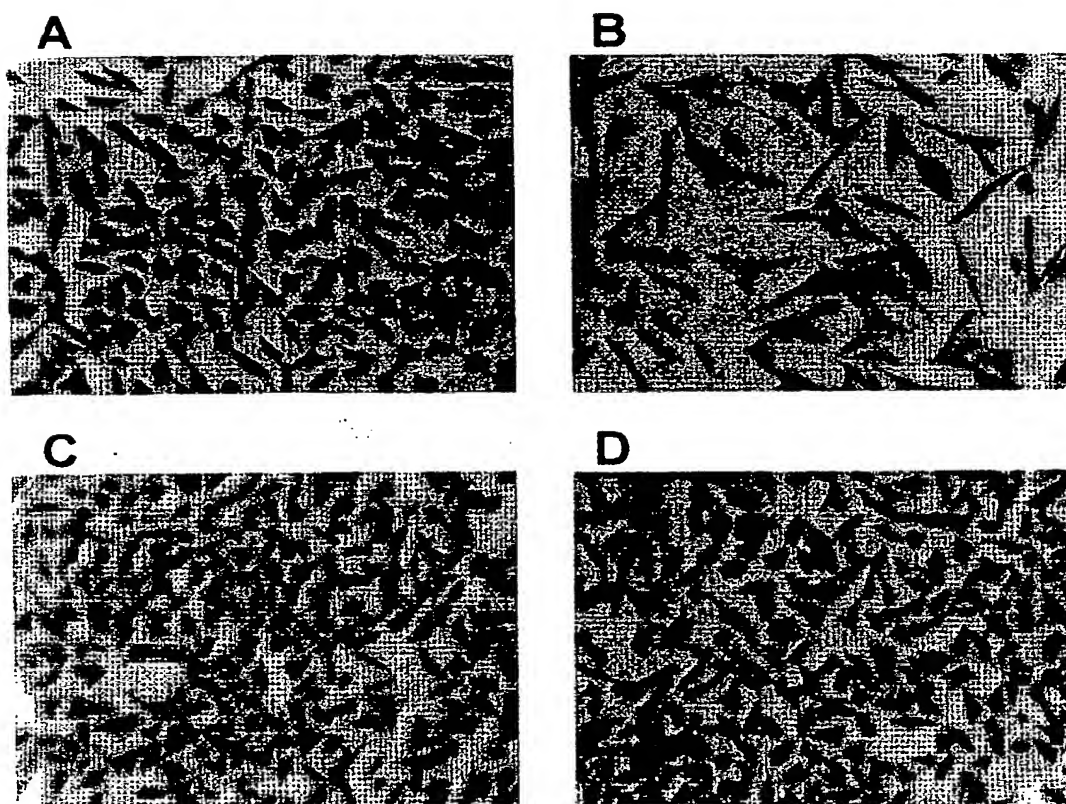
Fig. 2
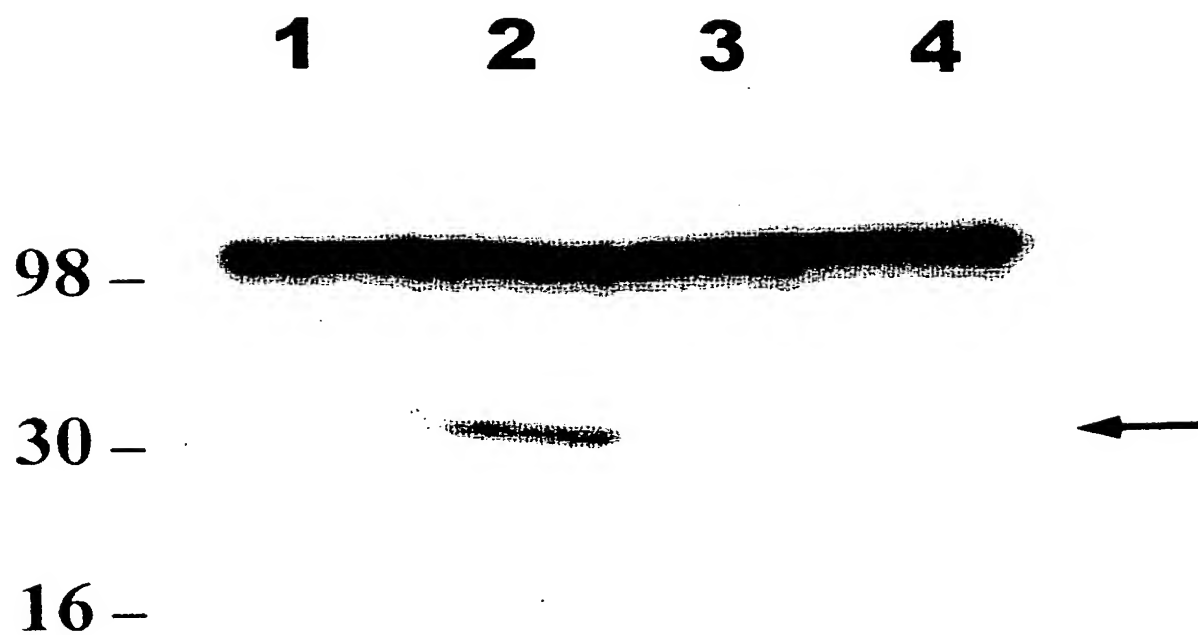
Fig. 3
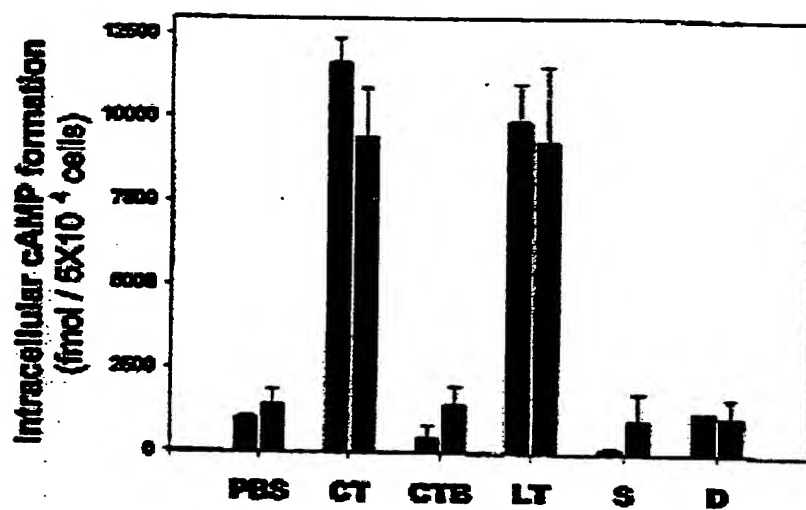
Fig. 4
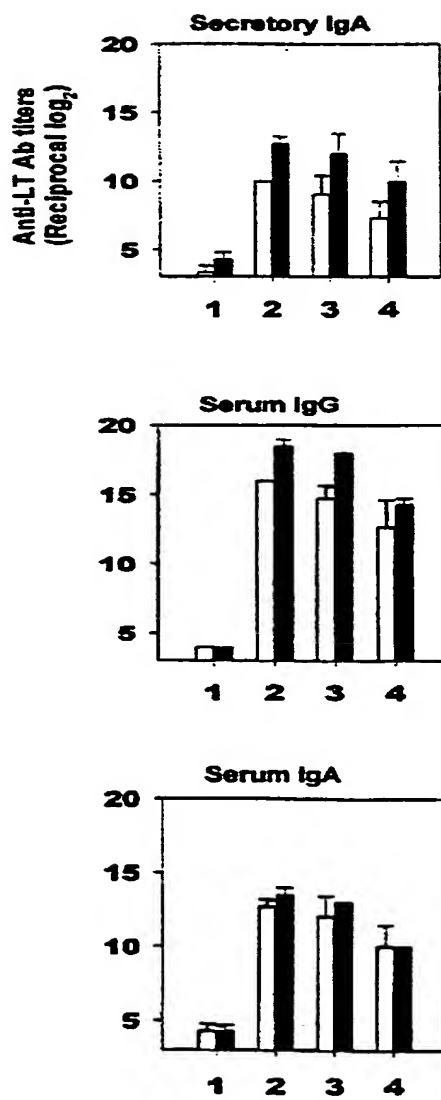
Fig. 5
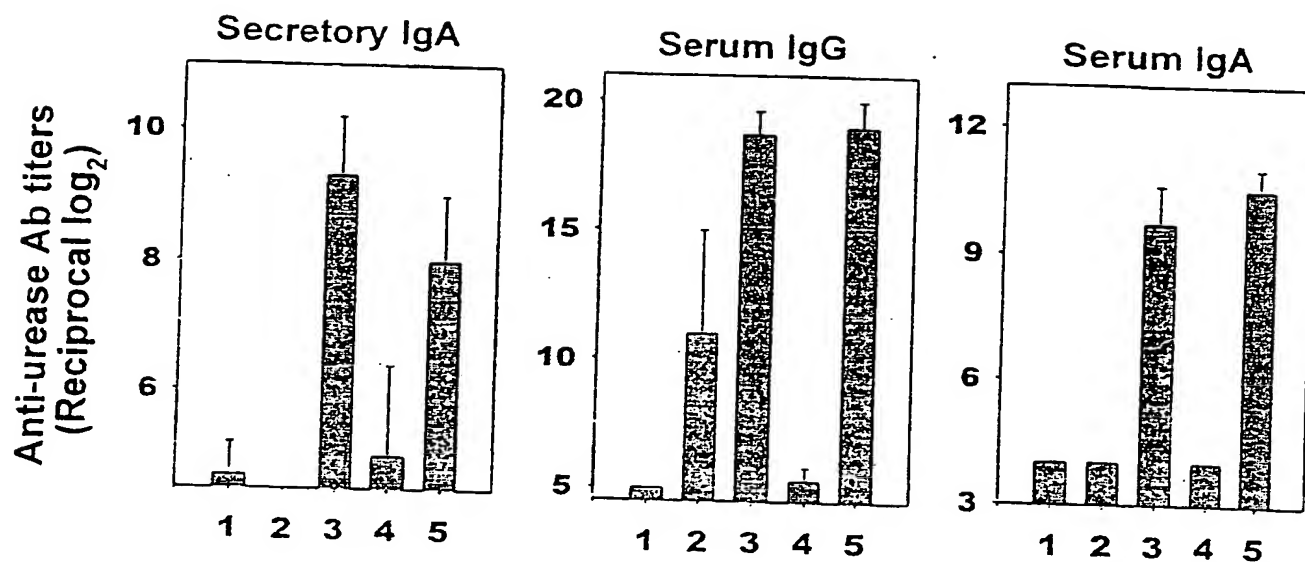
Fig. 6
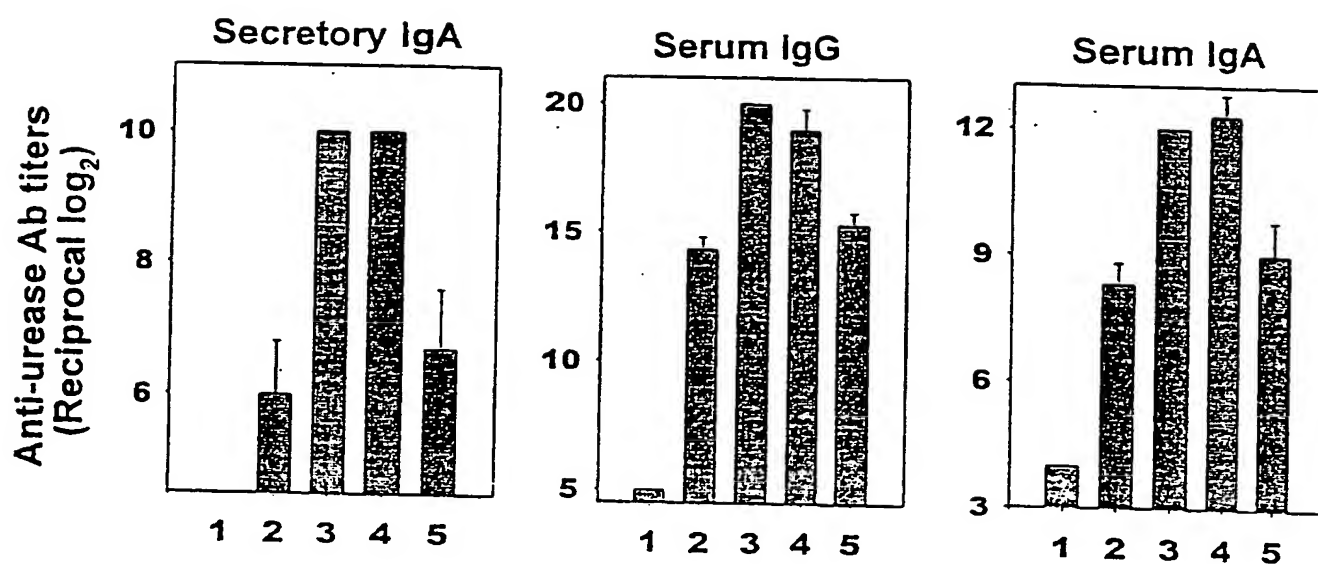
Fig. 7
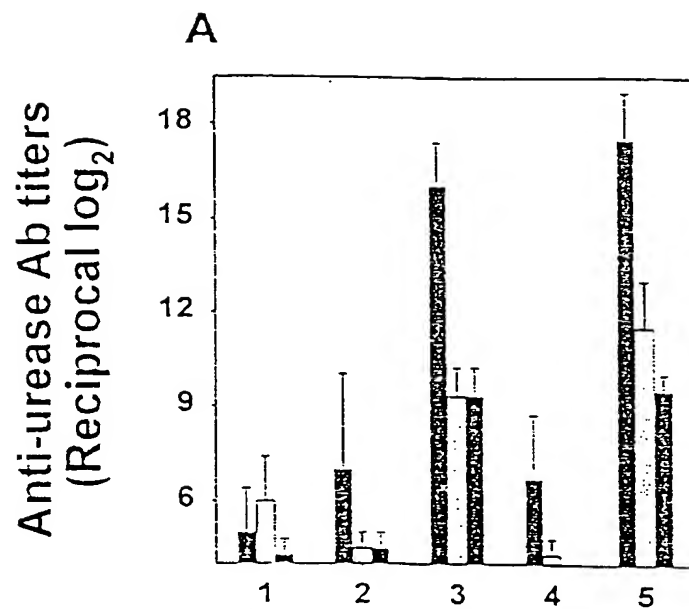
Fig. 8A
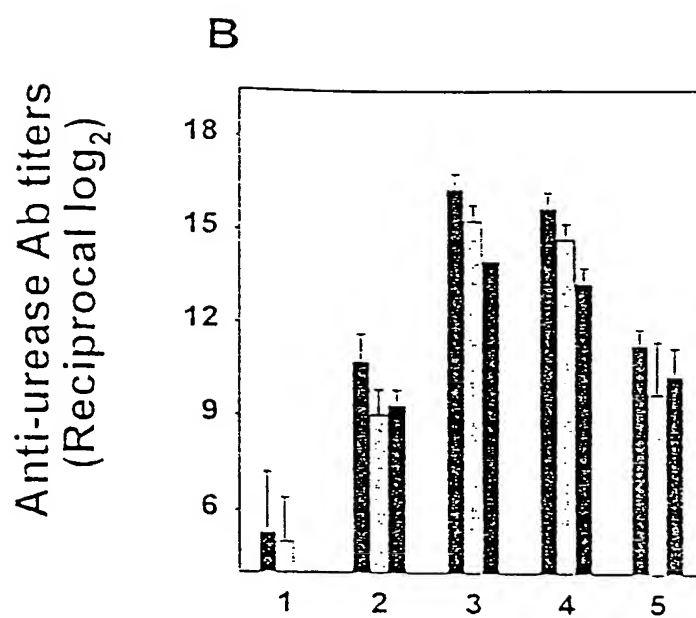
Fig. 8B

SEQUENCE LISTING

<110>   MOGAM BIOTECHNOLOGY RESEARCH INSTITUTE et al.

<120>   NOVEL DETOXIFITED MUTANTS OF Escherichia coli HEAT-LABILE ENTEROT OXIN

<160>   6

<170>   KOPATIN 1.5

<210>   1
<211>   52
<212>   DNA
<213>   Artificial Sequence

<220>
<223>   single-stranded nucleotide

<400>   1

atatgatgac ggatatgttt ccacttacct tagtttgaga agtgctcact tg   52

<210>   2
<211>   53
<212>   DNA
<213>   Artificial Sequence

<220>
<223>   single-stranded oligonucleotide

<400>   2

aggcgtatac agccctcacc catatcaggt ttctgcgtta ggtggaatac cat   53

<210>   3

2

<211>   382
<212>   PRT
<213>   Escherichia coli

<400>   3
Met Lys Asn Ile Thr Phe Ile Phe Phe Ile Leu Leu Ala Ser Pro Leu
 1   5   10   15

Tyr Ala Asn Gly Asp Arg Leu Tyr Arg Ala Asp Ser Arg Pro Pro Asp
            20   25   30

Glu Ile Lys Arg Ser Gly Gly Leu Met Pro Arg Gly His Asn Glu Tyr
            35   40   45

Phe Asp Arg Gly Thr Gln Met Asn Ile Asn Leu Tyr Asp His Ala Arg
            50   55   60

Gly Thr Gln Thr Gly Phe Val Arg Tyr Asp Asp Gly Tyr Val Ser Thr
65   70   75   80

Tyr Leu Ser Leu Arg Ser Ala His Leu Ala Gly Gln Ser Ile Leu Ser
                85   90   95

Gly Tyr Ser Thr Tyr Tyr Ile Tyr Val Ile Ala Thr Ala Pro Asn Met
                100   105   110

Phe Asn Val Asn Asp Val Leu Gly Val Tyr Ser Pro His Pro Tyr Glu
                115   120   125

Gln Glu Val Ser Ala Leu Gly Gly Ile Pro Tyr Ser Gln Ile Tyr Gly
                130   135   140

Trp Tyr Arg Val Asn Phe Gly Val Ile Asp Glu Arg Leu His Arg Asn
145   150   155   160

Arg Glu Tyr Arg Asp Arg Tyr Tyr Arg Asn Leu Asn Ile Ala Pro Ala
                165   170   175

Glu Asp Gly Tyr Arg Leu Ala Gly Phe Pro Pro Asp His Gln Ala Trp
            180   185   190

5   Arg Glu Glu Pro Trp Ile His His Ala Pro Gln Gly Cys Gly Asn Ser
            195   200   205

    Ser Arg Thr Ile Thr Gly Asp Thr Cys Asn Glu Glu Thr Gln Asn Leu
            210   215   220

10
    Ser Thr Ile Tyr Leu Arg Glu Tyr Gln Ser Lys Val Lys Arg Gln Ile
        225   230   235   240

    Phe Ser Asp Tyr Gln Ser Glu Val Asp Ile Tyr Asn Arg Ile Arg Asp
15   245   250   255

    Glu Leu Met Asn Lys Val Lys Phe Tyr Val Leu Phe Thr Ala Leu Leu
            260   265   270

20 Ser Ser Leu Cys Ala His Gly Ala Pro Gln Ser Ile Thr Glu Leu Cys
            275   280   285

    Ser Glu Tyr His Asn Thr Gln Ile Tyr Thr Ile Asn Asp Lys Ile Leu
        290   295   300

25
    Ser Tyr Thr Glu Ser Met Ala Gly Lys Arg Glu Met Val Ile Ile Thr
    305   310   315   320

    Phe Lys Ser Gly Ala Thr Phe Gln Val Glu Val Pro Gly Ser Gln His
30   325   330   335

    Ile Asp Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg
            340   345   350

35 Ile Thr Tyr Leu Thr Glu Thr Lys Ile Asp Lys Leu Cys Val Trp Asn
            355   360   365

Asn Lys Thr Pro Asn Ser Ile Ala Ala Ile Ser Met Glu Asn
      370   375   380

 5   <210>   4
      <211>   1514
      <212>   DNA
      <213>   Escherichia coli

10   <400>   4
      ggatccgtgc actctttctt tatcgcttca ctacacattt tatcctcgca tggatgtttt   60

      ataaaaaaca tgattgacat catgttgcat ataggttaaa caaaacaagt ggcgttatct   120

15   ttttccggat tgtcttcttg tatgatatat aagttttcct cgaatgaaaa atataacttt   180

      catttttttt attttattag catcgccatt atatgcaaat ggcgacagat tataccgtgc   240

      tgactctaga cccccagatg aaataaaaacg ttccggaggt cttatgccca gagggcataa   300
20

      tgagtacttc gatagaggaa ctcaaatgaa tattaatctt tatgatcacg cgagaggaac   360

      acaaaccggc tttgtcagat atgatgacgg atatgtttcc acttacctta gtttgagaag   420

25   tgctcactta gcaggacagt ctatattatc aggatattcc acttactata tatatgttat   480

      agcgacagca ccaaatatgt ttaatgttaa tgatgtatta ggcgtataca gccctcaccc   540

      atatgaacag gaggtttctg cgttaggtgg aataccatat tctcagatat atggatggta   600
30

      tcgtgttaat tttggtgtga ttgatgaacg attacatcgt aacagggaat atagagaccg   660

      gtattacaga aatctgaata tagctccggc agaggatggt tacagattag caggtttccc   720

35   accggatcac caagcttgga gagaagaacc ctggattcat catgcaccac aaggttgtgg   780

      aaattcatca agaacaatca caggtgatac ttgtaatgag gagacccaga atctgagcac   840

5

aatatatctc agggaatatc aatcaaaagt taagaggcag atattttcag actatcagtc   900

agaggttgac atatataaca gaattcggga tgaattatga ataaagtaaa attttatgtt   960

ttatttacgg cgttactatc ctctctatgt gcacacggag ctcctcagtc tattacagaa   1020

ctatgttcgg aatatcacaa cacacaaata tatacgataa atgacaagat actatcatat   1080

acggaatcga tggcaggcaa aagagaaatg gttatcatta catttaagag cggcgcaaca   1140

tttcaggtcg aagtcccggg cagtcaacat atagactccc aaaaaaaagc cattgaaagg   1200

atgaaggaca cattaagaat cacatatctg accgagacca aaattgataa attatgtgta   1260

tggaataata aaaccccccaa ttcaattgcg gcaatcagta tggaaaacta gtttgcttta   1320

aaagcatgtc taatgctagg aacctatata acaactactg tacttatact aatgagcctt   1380

atgctgcatt tgaaaaggcg gtagaggatg caataccgat ccttaaactg taacactata   1440

acagcttcca ctacagggag ctgttatagc aaacagaaaa aactaagcta ggctggaggg   1500

gcaagcttgg atcc   1514

<210>   5
<211>   380
<212>   PRT
<213>   Escherichia coli

<400>   5
Met Lys Asn Ile Thr Phe Ile Phe Phe Ile Leu Leu Ala Ser Pro Leu
1   5   10   15

Tyr Ala Asn Gly Asp Arg Leu Tyr Arg Ala Asp Ser Arg Pro Pro Asp
            20   25   30

Glu Ile Lys Arg Ser Gly Gly Leu Met Pro Arg Gly His Asn Glu Tyr
      35   40   45

5   Phe Asp Arg Gly Thr Gln Met Asn Ile Asn Leu Tyr Asp His Ala Arg
        50   55   60

Gly Thr Gln Thr Gly Phe Val Arg Tyr Asp Asp Gly Tyr Val Ser Thr
     65   70   75   80

10

Ser Leu Ser Leu Arg Ser Ala His Leu Ala Gly Gln Ser Ile Leu Ser
          85   90   95

Gly Tyr Ser Thr Tyr Tyr Ile Tyr Val Ile Ala Thr Ala Pro Asn Met
15   100   105   110

Phe Asn Val Asn Asp Val Leu Gly Val Tyr Ser Pro His Pro Tyr Gln
        115   120   125

20   Val Ser Ala Leu Gly Gly Ile Pro Tyr Ser Gln Ile Tyr Gly Trp Tyr
       130   135   140

Arg Val Asn Phe Gly Val Ile Asp Glu Arg Leu His Arg Asn Arg Glu
    145   150   155   160

25

Tyr Arg Asp Arg Tyr Tyr Arg Asn Leu Asn Ile Ala Pro Ala Glu Asp
       165   170   175

Gly Tyr Arg Leu Ala Gly Phe Pro Pro Asp His Gln Ala Trp Arg Glu
30   180   185   190

Glu Pro Trp Ile His His Ala Pro Gln Gly Cys Gly Asn Ser Ser Arg
      195   200   205

35   Thr Ile Thr Gly Asp Thr Cys Asn Glu Glu Thr Gln Asn Leu Ser Thr
      210   215   220

Ile Tyr Leu Arg Glu Tyr Gln Ser Lys Val Lys Arg Gln Ile Phe Ser
225   230   235   240

Asp Tyr Gln Ser Glu Val Asp Ile Tyr Asn Arg Ile Arg Asp Glu Leu
                    245   250   255

Met Asn Lys Val Lys Phe Tyr Val Leu Phe Thr Ala Leu Leu Ser Ser
                    260   265   270

Leu Cys Ala His Gly Ala Pro Gln Ser Ile Thr Glu Leu Cys Ser Glu
                275   280   285

Tyr His Asn Thr Gln Ile Tyr Thr Ile Asn Asp Lys Ile Leu Ser Tyr
        290   295   300

Thr Glu Ser Met Ala Gly Lys Arg Glu Met Val Ile Ile Thr Phe Lys
305   310   315   320

Ser Gly Ala Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp
                    325   330   335

Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Thr
                340   345   350

Tyr Leu Thr Glu Thr Lys Ile Asp Lys Leu Cys Val Trp Asn Asn Lys
                355   360   365

Thr Pro Asn Ser Ile Ala Ala Ile Ser Met Glu Asn
        370   375   380

<210>   6
<211>   1508
<212>   DNA
<213>   Escherichia coli

<400>   6

8

ggatccgtgc actctttctt tatcgcttca ctacacattt tatcctcgca tggatgtttt   60

ataaaaaaca tgattgacat catgttgcat ataggttaaa caaaacaagt ggcgttatct   120

5   ttttccggat tgtcttcttg tatgatatat aagttttcct cgaatgaaaa atataacttt   180

catttttttt attttattag catcgccatt atatgcaaat ggcgacagat tataccgtgc   240

tgactctaga cccccagatg aaataaaacg ttccggaggt cttatgccca gagggcataa   300

10   tgagtacttc gatagaggaa ctcaaatgaa tattaatctt tatgatcacg cgagaggaac   360

acaaaccggc tttgtcagat atgatgacgg atatgtttcc acttctctta gtttgagaag   420

15   tgctcactta gcaggacagt ctatattatc aggatattcc acttactata tatatgttat   480

agcgacagca ccaaatatgt ttaatgttaa tgatgtatta ggcgtataca gccctcaccc   540

atatcaggtt tctgcgttag gtggaatacc atattctcag atatatggat ggtatcgtgt   600

20   taattttggt gtgattgatg aacgattaca tcgtaacagg gaatatagag accggtatta   660

cagaaatctg aatatagctc cggcagagga tggttacaga ttagcaggtt tcccaccgga   720

25   tcaccaagct tggagagaag aaccctggat tcatcatgca ccacaaggtt gtggaaattc   780

atcaagaaca atcacaggtg atacttgtaa tgaggagacc cagaatctga gcacaatata   840

tctcagggaa tatcaatcaa aagttaagag gcagatattt tcagactatc agtcagaggt   900

30   tgacatatat aacagaattc gggatgaatt atgaataaag taaaatttta tgttttattt   960

acggcgttac tatcctctct atgtgcacac ggagctcctc agtctattac agaactatgt   1020

35   tcggaatatc acaacacaca aatatatacg ataaatgaca agatactatc atatacggaa   1080

tcgatggcag gcaaaagaga aatggttatc attacattta agagcggcgc aacatttcag   1140

gtcgaagtcc cgggcagtca acatatagac tcccaaaaaa aagccattga aaggatgaag   1200

gacacattaa gaatcacata tctgaccgag accaaaattg ataaattatg tgtatggaat   1260

5

aataaaaccc ccaattcaat tgcggcaatc agtatggaaa actagtttgc tttaaaagca   1320

tgtctaatgc taggaaccta tataacaact actgtactta tactaatgag ccttatgctg   1380

10   catttgaaaa ggcggtagag gatgcaatac cgatccttaa actgtaacac tataacagct   1440

tccactacag ggagctgtta tagcaaacag aaaaaactaa gctaggctgg aggggcaagc   1500

ttggatcc   1508

INTERNATIONAL SEARCH REPORT

CLASSIFICATION OF SUBJECT MATTER

IPC[7]: C 12 N 15/31, 15/09, 1/21; A 61 K 39/108 // (C 12 N 1/21, C 12 R 1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[7]: C 12 N 15/31, 15/09, 1/21; A 61 K 39/108

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAS, STN-registry

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0620850 B1 (CHIRON S.P.A.) 3 March 1999 (03.03.99) page 2, lines 17-25; claims 1,3,5,11,19.	1,10,12,13
A	WO 96/06627 A1 (THE ADMINISTRATORS OF THE TULANE EDUCATIONAL FUND) 7 March 1996 (07.03.96) amended claims 1,4-12,14-17,19,23-25.	1-13
A	WO 98/28005 A1 (SMITHKLINE BEECHAM BIOLOGICALS S.A.) 2 July 1998 (02.07.98) claims 1,2,6,7,9.	1,13

☐ Further documents are listed in the continuation of Box C.   ☒ See patent family annex.

* Special categories of cited documents:
„A" document defining the general state of the art which is not considered to be of particular relevance
„E" earlier application or patent but published on or after the international filing date
„L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
„O" document referring to an oral disclosure, use, exhibition or other means
„P" document published prior to the international filing date but later than the priority date claimed

„T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
„X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
„Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
„&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 May 2000 (17.05.2000)	2 August 2000 (02.08.2000)

Name and mailing adress of the ISA/AT	Authorized officer
Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna	Mosser
Facsimile No. 1/53424/535	Telephone No. 1/53424/437

Form PCT/ISA/210 (second sheet) (July 1998)

Patent document cited in search report			Publication date	Patent family member(s)			Publication date
EP	A1	620850	26-10-1994	AT	E	177145	15-03-1999
EP	B1	620850	03-03-1999	AU	A1	33476/93	28-07-1993
				DE	C0	69228563	08-04-1999
				DE	T2	69228563	29-07-1999
				EP	A1	869181	07-10-1998
				ES	T3	2127808	01-05-1999
				GR	T3	3029556	30-06-1999
				IT	A0	913513	31-12-1991
				IT	A	1253009	10-07-1995
				JP	T2	7506240	13-07-1995
				MX	A1	9207685	31-05-1994
				SG	A1	48217	17-04-1998
				WO	A1	9313202	08-07-1993
WO	A1	9606627	07-03-1996	AU	A1	32337/95	22-03-1996
				AU	B2	709779	09-09-1999
				BR	A	9508633	30-09-1997
				CA	AA	2198586	07-03-1996
				CN	A	1164191	05-11-1997
				CZ	A3	9700562	15-10-1997
				EP	A1	777490	11-06-1997
				EP	A4	777490	18-11-1998
				FI	A0	970799	26-02-1997
				FI	A	970799	24-04-1997
				HU	A2	77869	28-09-1998
				JP	T2	10505059	19-05-1998
				NO	A0	970993	04-03-1997
				NO	A	970993	25-04-1997
				NZ	A	291262	25-02-1999
				PL	A1	318930	21-07-1997
				US	A	6019982	01-02-2000
				ZA	A	9506412	11-03-1996
WO	A1	9828005	02-07-1998	AU	A1	57639/98	17-07-1998
				EP	A1	948352	13-10-1999
				GB	A0	9626864	12-02-1997
				ZA	A	9711565	27-08-1998